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<b>(21) International Application Number:</b> PCT/US90/07348 <b>(22) International Filing Date:</b> 13 December 1990 (13.12.90)  <b>(30) Priority data:</b> 466,981 18 January 1990 (18.01.90) US  <b>(71) Applicant:</b> CETUS CORPORATION [US/US]; 1400 Fifty-Third Street, Emeryville, CA 94608 (US). <b>(72) Inventor:</b> WONG, Hing, Cheung ; 634 Lomond Circle, San Ramon, CA 94585 (US). <b>(74) Agent:</b> MCGARRIGLE, Philip, L., Jr.; Cetus Corporation, 1400 Fifty-Third Street, Emeryville, CA 94608 (US).		<b>(81) Designated States:</b> AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> IMPROVED STREPTOMYCES PROMOTER  <b>(57) Abstract</b>  The present invention is a DNA sequence comprising a Streptomyces thiostrepton promoter (tipA) wherein a base, preferably guanine, is inserted between positions -50 and -75, preferably positions -62 and -63. This promoter is stronger than the wild type and ultimately increases transcription initiation and protein expression.		

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## IMPROVED STREPTOMYCES PROMOTER

The present invention relates to a Streptomyces promoter. More specifically, the present invention is an improved promoter which is useful to ultimately increase expression and efficiency of a gene.

5 A promoter is a DNA sequence that promotes gene transcription to produce a messenger RNA and is recognized by RNA polymerase, which the promoter directs to the correct transcriptional start site. The E. coli promoter region, which is about 50 bases long, includes a common sequence (Pribnow box) of about 6 bases located upstream (in the 5' direction) from the site at which RNA synthesis begins. Five to  
10 eight bases separate the Pribnow box from the initiation site, so that it centers around the -10 position. Not all E. coli promoters have exactly the same sequence of bases in this region, but a sequence similar to TATAAT is typically found. Another consensus sequence, TTGACA, centers around -35; this region is also critical for the accurate and rapid initiation of transcription for most bacterial genes (see Darnell et al., 1986,  
15 Molecular Cell Biology, Scientific American Books Inc.). It has been noted that the -10 and -35 regions, as well as the spacing between them, are "major determinants of promoter strength." Because of the conserved regions, the RNA polymerase appears to make direct contact with the two regions. Consequently, "most mutations that affect promoter strength map to the strongly conserved bases in the -10 or -35 region or  
20 change the spacing between them" (Jansen, et al., 1985, Microbiology, Meetings for the American Society for Microbiology, American Society for Microbiology, Washington, D.C., pages 392-396 [1985]).

Mutations in the promoter may effect initiation. It has been shown that changes, deletions, or insertions in the promoter region may effect the way that RNA  
25 polymerase binds to the promoter. The ultimate result may be that more or less mRNA is produced. For example, Mandecki et al., 1982, Nucleic Acid Research 10:903-912, show that a two base pair insertion between the -10 and -35 regions lowered the promoter activity to 15% of the wild type; Post et al., 1978, Cell 15:231-236, show that a point mutation six bases upstream from the in vitro transcription site, which changed the Pribnow box, was important in the expression of an operon; and Youderian et al.,  
30 1982, Cell 30:843-853, show that specific decreases in the homology of a specific promoter with its consensus promoter sequence resulted in either a severe or a mild reduction in promoter activity (see Watson et al. Molecular Biology of Gene 4th edition Benjamin/Cummings Publishing Company, 1987). (Also see Botstein, 1972, Virology  
35 49:268-282, and Siebenlist et al., 1980, Cell 20:269-281.)

Since promoters are known to effect ultimate gene expression, it would advantageous to increase the strength of promoter by manipulating the promoter sequence. That is the focus of the present invention, which is detailed and described more fully below.

5       The present invention is a DNA sequence comprising a Streptomyces promoter, wherein a base is inserted into the base sequence between positions -50 and -75, to increase transcription initiation. Preferably, the base insertion is made between -60 and -65, more preferably between positions -62 and -63. Preferably the base is guanine. Preferably, the DNA sequence is regulated by thiostrepton and is operable in  
10 Streptomyces. Preferably, the DNA sequence will hybridize to the following DNA sequence: GCGGCGGCTCACGGGCGTGGCA. More preferably, the DNA sequence is shown in Figure 1. This improved DNA sequence may occur in the chromosome or may be in an expression vector. Preferably, the invention also includes a method for producing a protein using the DNA sequence detailed above.

15       The present inventor has made the surprising discovery that a base insertion between positions -62 and -63 of the Streptomyces thiostrepton promoter (tipA), increases promoter strength so that protein expression is increased 5-fold (while maintaining the regulatory aspects of the promoter). This is further surprising because the position of the mutation is outside the commonly recognized sequences for the  
20 promoter, i.e., near the -10 and -35 regions.

Figure 1 is the DNA sequence of the tipA wild type and mutant promoters.

Figure 2 is a diagram of the construction of pHC-1 and pHC-2.

Figure 3 is a circular map of pHC-2.

A promoter can be induced by a compound. In Streptomyces, thiostrepton is  
25 one such compound. Thiostrepton is an antibiotic which inhibits translation. However, it has been reported to induce a streptomyces promoter to express several proteins (Murakami *et al.*, 1989, Journal of Bacteriology 171(3):1459-1466, which is hereby incorporated by reference in its entirety). Murakami *et al.* show that thiostrepton induces the expression of four proteins of unknown function in Streptomyces lividans.  
30 Murakami *et al.* note that the promoter that is induced (tipA) should be useful for expression studies in streptomycetes. Since the streptomycetes are commonly used to commercially produce antibiotics, the tipA promoter can prove commercially useful. For example, certain genes can be operably linked to the thiostrepton promoter to increase their expression. These may be naturally occurring streptomyces genes (for  
35 example, antibiotic producing genes) or heterologous genes (for example, mammalian genes such as interleukins, interferons, colony stimulating factors, tumor necrosis factor, etc.). (See, the two articles collected in Microbiology, 1985, American Society

for Microbiology, Washington, D.C., pages 409-420, which are both hereby incorporated by reference in their entireties.) Preferably, the improved *tipA* promoter of the present invention is linked to a DNA sequence encoding a protein so that the protein may be controllably expressed. Preferably, other DNA control sequences are included that are necessary for production of the protein, such as ribosome binding sites, etc.

As stated above, the present invention is the discovery that inserting a guanine between positions -62 and -63 of the *tipA* promoter increases the strength of the promoter while maintaining the promoter control. This is an important discovery because this promoter may be useful to controllably overexpress various proteins made in streptomycetes. Currently, streptomycetes are used for pharmaceutical and antibiotic preparations. This invention may also be advantageous in further research into promoters.

Streptomyces are gram positive organisms with a complex cycle of morphological development. They can be grown from a spore on a solid surface to give rise to a branched septate substrate mycelium and aerial mycelium (see Hopwood *et al.*, Regulation of Gene Expression-25 Years On, The 39th Symposium of the Society for General Microbiology, Cambridge University Press 1986, pages 251-276, which is hereby incorporated by reference in its entirety). However, in submerged culture, the cells do not sporulate. After they complete the vegetative state, they remain biologically active and can produce large amounts of secondary metabolites, such as antibiotics. To illustrate this point of the 4,973 natural antibiotics described by 1,978, 2,769 were of streptomycetes origin. These antibiotics include aminoglycosides, macrolides, tetracyclines, polyethers, anasamycins,  $\beta$ -lactams, oligopeptides, etc. Streptomycetes can also produce other extracellular products, such as hydrolytic enzymes. See Chater *et al.*, 1982, Current Topics in Microbiology and Immunology 96:69-95, which is hereby incorporated by reference in its entirety. Although it is envisioned that the improved promoter can be inserted into all streptomycetes, exemplary hosts include the following: Streptomyces lividans, S. coelicolor, S. griseus, S. parvulus, S. albus, S. vinaceus, S. acrimycinis, S. calvuligerus, S. limosus, S. rubiginosis, S. azureus, S. glaucenscens, S. rimosus, S. violaceotuber, and S. kanamyceticus.

As stated above, an improved promoter is important because it can be linked to a gene of interest to increase the ultimate expression of that gene. The promoter can be operably linked to genes by means known in the art (see Maniatis *et al.*, 1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, and U.S. Serial No. 023,637, which are both herein incorporated by reference in their entireties).

Generally speaking, restriction sites may be introduced into the promoter and a specific gene of interest so that the two may be joined. Thereafter, the promoter and gene may be introduced into a vector, which may or may not be able to integrate into the streptomyces chromosome. A mechanism for inserting the promoter and/or the gene  
5 into the chromosome may be by use of a DNA sequence which favors integration. An example of this DNA sequence is the mini-circle DNA which is described below and in Lydiate *et al.*, 1986, *Mol. Gen. Genet.* 203:79-88, which is hereby incorporated by reference in its entirety.

The presently preferred *tipA* promoter differs from the wild type by one base.  
10 See Figure 1 which compares the two sequences. The wild type promoter is the uninterrupted sequence, and the improved promoter is that sequence plus the guanine that is inserted between the bases at positions -62 and -63. There are also some other structures of interest. The sequences that are labeled with "a" and "b" are direct repeats. The "a" sequence is found at the positions -95 to -81, -20 to -8, and 6 to 15.  
15 The "b" sequence is shown at positions -79 to -72 and -58 to -50. There are also two inverted repeats, which occur at positions -48 to -26 and -2 to -22.

Even though the most preferred embodiment is shown in Figure 1, other sequences may increase promoter strength and, therefore, transcription initiation. For example, other bases may be inserted in lieu of guanine, such as thymine, adenine, or  
20 cytosine. Also, the insertion may be made at points other than between -62 and -63. The insertion could occur somewhere between -60 and -65, or even between -50 and -75. Furthermore, increased initiation may occur if the sequence shown between -55 and -75 on Figure 1 is shifted to a different position within the promoter. However, the shifted sequence should remain substantially homologous to the following  
25 sequence: GCGGCGGCTCACGGGCGTGGCA (the underlining denotes the inserted guanine).

The present invention will now be illustrated by reference to the following examples, which set forth the particularly advantageous embodiments. However, it should be noted that these embodiments are illustrative and are not to be construed as a  
30 restricting the invention in any way.

#### Example 1

##### Construction of an Integrated Promoter-Probe Plasmid

The integrated promoter-probe plasmid was constructed to include the following components: an *E. coli* replication origin (*colEI* replication origin from the pUC18); an  
35 *E. coli* selectable marker (Tn3  $\beta$ -lactamase); an indicator gene (catechol dioxygenase, designated XylE); a *Streptomyces* selectable marker (hygromycin resistance gene); a

Streptomyces integration sequence (mini-circle DNA); and a linker region containing specific restriction sites (HindIII, KpnI, SmaI, BamHI, and XbaI). The general sequence for this plasmid's construction is shown in Figure 2 and is recited below.

The plasmid pUC18 (commercially obtained) was partially digested with PvuII and then digested with EcoRI. The EcoRI restriction ends were repaired with DNA polymerases I fragment and four deoxyribonucleotide triphosphates. After the restriction ends were filled in, the plasmid was religated and transformed into E. coli DG101. Plasmids from ampicillin resistant transformants were isolated and analyzed. These plasmids were designated pEM3 (pUC18 without the lac promoter). Plasmid pEM3 was ligated with DNA sequences from the plasmids pIJ2349 (hygromycin resistance) and pIJ4210 (mini-circle DNA). Plasmids pIJ4210 and pIJ2349 were obtained from John Innes Institute, Norwich NR7UH, United Kingdom. Further information can be found in Chater *et al.*, 1982, Current Topics in Microbiology and Immunology 26:69-95, for the pIJ101 series of plasmids and Hopwood *et al.*, Genetic Manipulation of Streptomyces, a laboratory manual, The John Innes Foundation, Norwich, United Kingdom. The hygromycin resistance gene was obtained by digesting pIJ2349 to obtain a 1.0 kb fragment. The mini-circle DNA was obtained by digesting pIJ4210 to obtain a 2.10 kb fragment (see Lydiate *et al.* referred to above). After digestion with EcoRI and PstI, pEM3 was joined to the fragments from pIJ2349 and pIJ4210 in a three fragment ligation to yield pHC-I. Plasmid pIJ4081 was obtained from the John Innes Institute and was digested as shown in Figure 2. After digestion with restriction enzymes, a 1.5 kb fragment containing the Xyle and the linker region was obtained and ligated to the fragment generated from pHC-I, as also shown in Figure 2. The resulting plasmid was designated pHC-2.

## Example 2

### Cloning of the tipA Promoter

The tipA promoter was constructed using two overlapping oligonucleotides, a 77 and 78 base primer, as shown as follows:

77-mer CCAAGCTTCC CGGGCTGAGG GAGCCGACGG CACGCGGCGG CTCACGGCGT GGCACGCGGA  
ACGTCGGGGC TTGCACC  
78-mer CCTCTAGACT TCGCTCCCT TCTCTGACGC CGTCCACGCT GCCTCCTCAC GTGACGTGAG  
GTGCAAGCCC GGACGTTT

These two primers were annealed and extended by DNA polymerase I large fragment and deoxyribonucleotides. After extension, the double-stranded DNA was digested with HindIII and XbaI. The digested DNA was cloned into the HindIII-XbaI site of pUC18. The recombinant plasmid was sequenced. It contained the improved promoter

having the guanine insertion between bases -62 and -63. The promoter was designated tipA-VG62. The wild type and mutant promoters were subcloned into the promoter-probe plasmid pHC-2. The plasmid having the wild type promoter was designated pHC11-11, and the plasmid having the improved promoter was designated pHC11-3.

5

### Example 3

#### tipA Promoter Activity and Streptomyces lividans

Both plasmids were then transformed into Streptomyces lividans. Cultures of Streptomyces lividans were grown and the specific activity of the XylE gene was analyzed in the presence and absence of thiostrepton (thiostrepton induces the tipA promoter to transcribe the XylE gene). The results of this experiment are shown in Table I.

10

Table I

#### tipA Promoter Activity in Streptomyces lividans

<u>Construct</u>	<u>Promoter</u>	<u>Specific Activity of XylE*</u>	
		<u>-Thiostrepton</u>	<u>+Thiostrepton</u>
15 pHC11-11	<u>tipA-wt</u>	< 0.5	36.3
pHC11-3	<u>tipA-VG62</u>	12.0	182.2

\* Specific activity is defined as mmoles of catechol semialdehyde formed per minute per mg of protein.

20 The results show that there was 5-fold more protein produced from the plasmid having the improved promoter.

The plasmid pHC11-3 has been deposited in Cetus' in-house depository as CMCC 3798 and in the ATCC as 40737.

25 - The present invention has been described with reference to specific embodiments. However, this application is intended to cover those changes and substitutions which may be made by those skilled in art without departing from the spirit and the scope of the appended claims.



In the Claims

1. A DNA sequence comprising a Streptomyces promoter, wherein a base is inserted into the base sequence between positions -50 and -75, to increase transcription initiation.
- 5 2. A DNA sequence in accordance with Claim 1, wherein a base is inserted into the base sequence between positions -60 and -65, to increase transcription initiation.
3. A DNA sequence comprising a Streptomyces promoter, wherein a guanine is inserted into the base sequence between positions -62 and -63.
- 10 4. A DNA sequence in accordance with Claim 3, wherein the DNA sequence is regulated by thiostrepton.
5. A DNA sequence that hybridizes to the following DNA sequence:  
GCGGCGGCTCACGGGCGTGGCA and which increases transcription initiation  
when placed upstream of a gene.
- 15 6. A DNA sequence in accordance with Claim 5 comprising the base sequence shown in Figure 1, from positions -1 to -100, including the guanine insertion between -62 and -63.
7. A DNA sequence in accordance with Claim 6 further comprising operably linking a gene to the DNA sequence to control the expression of the gene.
- 20 8. A DNA sequence in accordance with Claim 7, wherein the promoter and gene are contained in an expression vector.
9. A DNA sequence in accordance with Claim 7, wherein the promoter and gene are contained in the chromosome.
- 25 10. A DNA sequence in accordance with Claim 7, wherein the gene is a heterologous gene.

11. A DNA sequence in accordance with Claim 10, wherein the gene encodes for a protein selected from the group consisting essentially of interleukins, interferons, colony stimulating factors, and tumor necrosis factor.

12. A DNA sequence is accordance with Claim 7, wherein the gene is found in  
5 Streptomyces.

13. A DNA sequence in accordance with Claim 12, wherein the gene encodes for a protein selected from the group of antibiotics consisting essentially of aminoglycosides, macrolides, tetracyclines, polyethers, anasamycins,  $\beta$ -lactams, and oligopeptides.

10 14. A method for producing a protein comprising operably linking a gene encoding the protein to the DNA sequence of Claim 1 and expressing the protein.

15. A method for producing a protein comprising operably linking a gene encoding the protein to the DNA sequence of Claim 3 and expressing the protein.

15 16. A method for producing a protein comprising operably linking a gene encoding the protein to the DNA sequence of Claim 6 and expressing the protein.

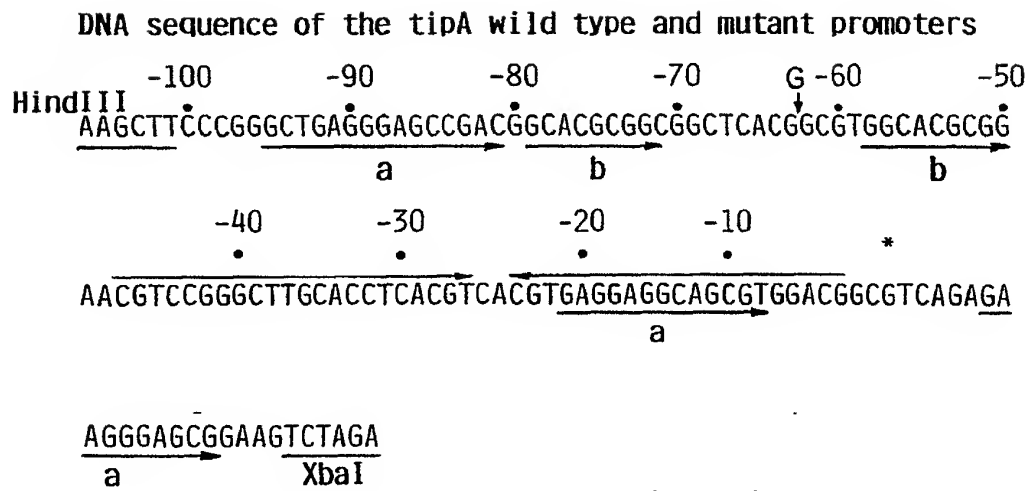


FIG. 1

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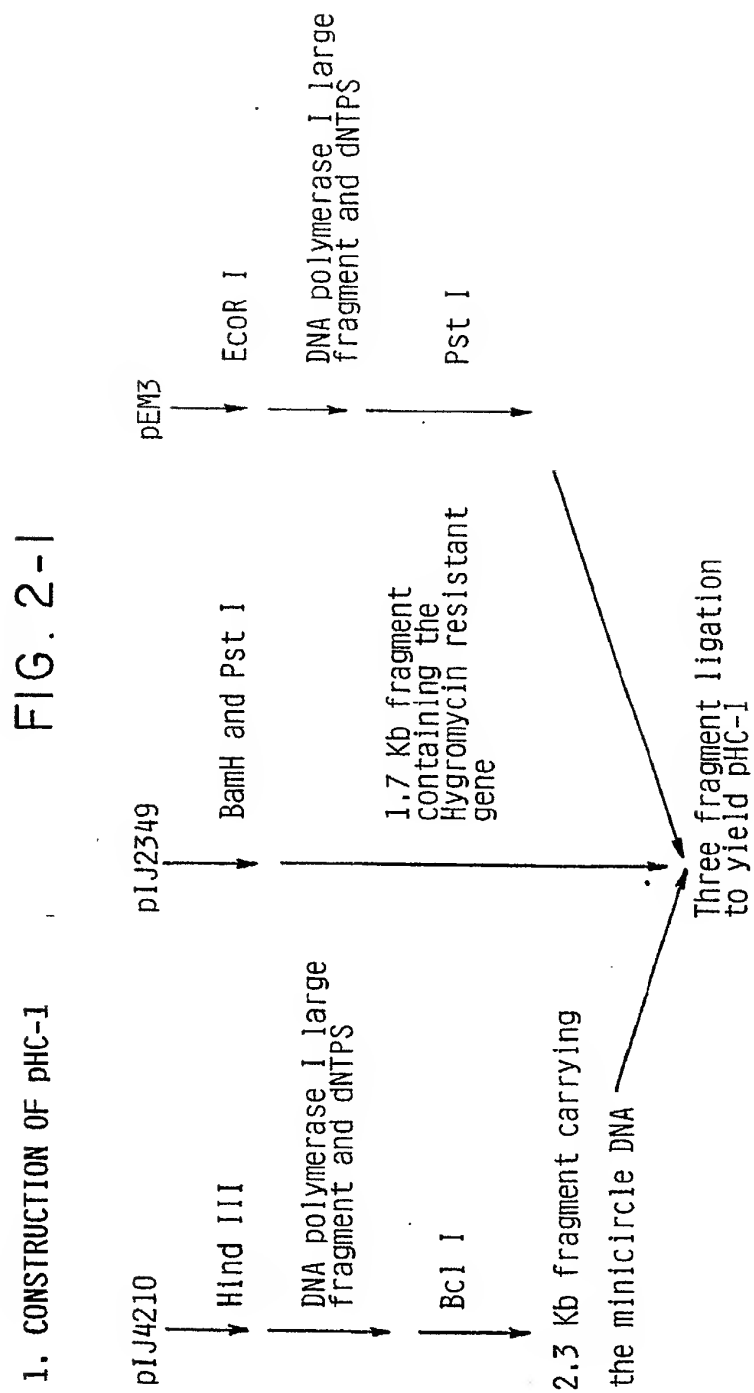
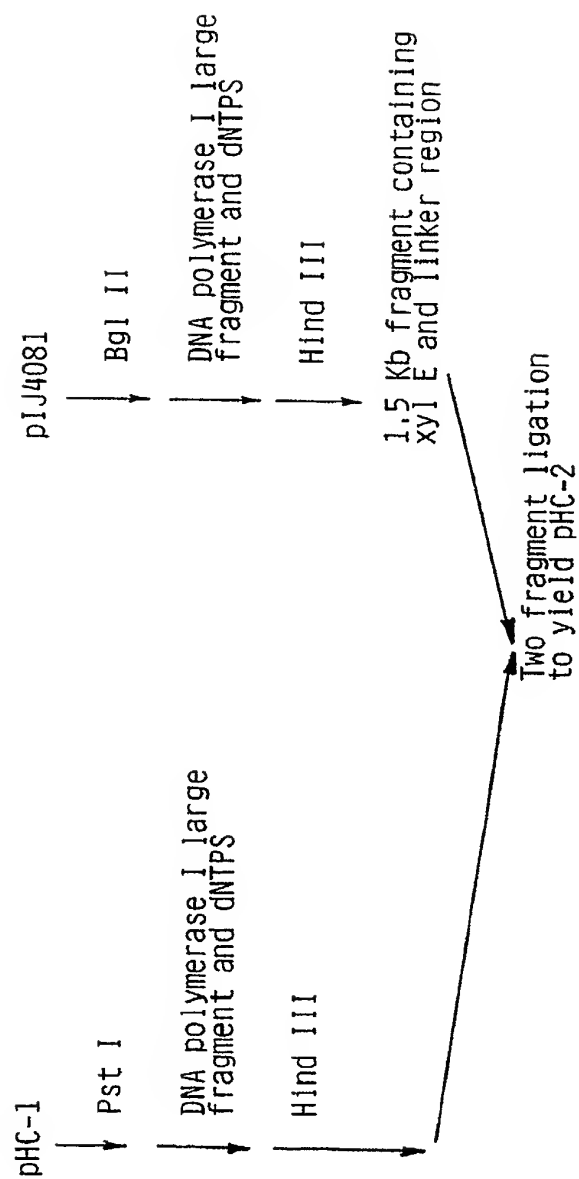
**SUBSTITUTE SHEET**

FIG. 2-2

2. Construction of pHc-2



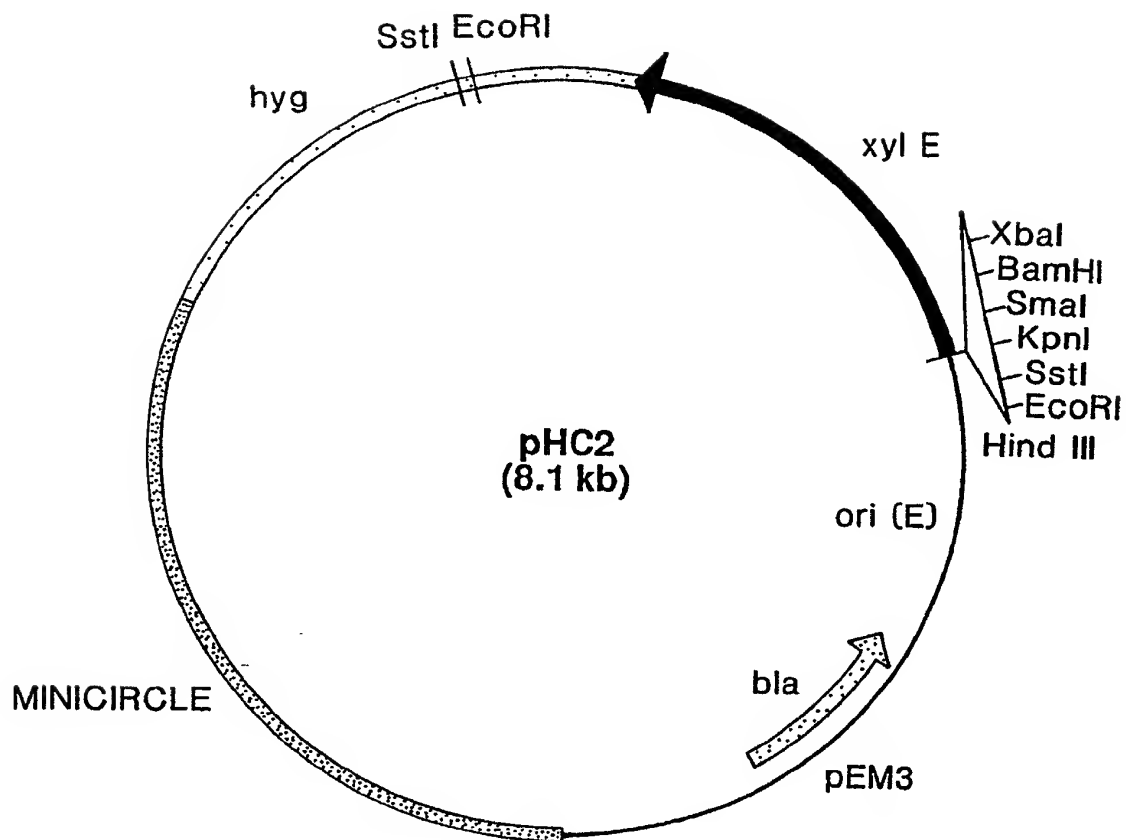
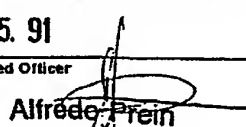
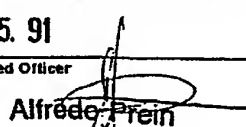
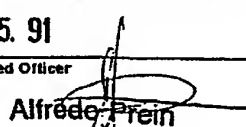


FIG. 3

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# INTERNATIONAL SEARCH REPORT

International Application No PCT/US 90/07348

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>6</sup> According to International Patent Classification (IPC) or to both National Classification and IPC IPC5: C 12 N 15/76										
<b>II. FIELDS SEARCHED</b> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Minimum Documentation Searched<sup>7</sup></div> <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 25%; border-bottom: 1px solid black;">Classification System</td> <td style="border-bottom: 1px solid black;">Classification Symbols</td> </tr> <tr> <td style="height: 40px; vertical-align: bottom;">IPC5</td> <td style="vertical-align: bottom;">C 12 N</td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Documentation Searched other than Minimum Documentation to the extent that such Documents are included in Fields Searched<sup>8</sup></div>			Classification System	Classification Symbols	IPC5	C 12 N				
Classification System	Classification Symbols									
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<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 10%; border-bottom: 1px solid black;">Category *</th> <th style="width: 60%; border-bottom: 1px solid black;">Citation of Document,<sup>11</sup> with indication, where appropriate, of the relevant passages<sup>12</sup></th> <th style="width: 30%; border-bottom: 1px solid black;">Relevant to Claim No.13</th> </tr> <tr> <td style="vertical-align: top; border-right: 1px solid black; height: 300px;">A</td> <td style="border-right: 1px solid black; vertical-align: top;">           JOURNAL OF BACTERIOLOGY, vol. 171, No. 3, 1989, T. Murakami et al: "Thiostrepton-Induced Gene Expression in Streptomyces lividans ", see page 1459 - page 1466   <div style="text-align: center;">             ---              -----           </div> </td> <td style="vertical-align: top;">1-16</td> </tr> </table>			Category *	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No.13	A	JOURNAL OF BACTERIOLOGY, vol. 171, No. 3, 1989, T. Murakami et al: "Thiostrepton-Induced Gene Expression in Streptomyces lividans ", see page 1459 - page 1466  <div style="text-align: center;">             ---              -----           </div>	1-16		
Category *	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No.13								
A	JOURNAL OF BACTERIOLOGY, vol. 171, No. 3, 1989, T. Murakami et al: "Thiostrepton-Induced Gene Expression in Streptomyces lividans ", see page 1459 - page 1466  <div style="text-align: center;">             ---              -----           </div>	1-16								
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p><sup>*</sup> Special categories of cited documents: <sup>10</sup></p> <p><sup>"A"</sup> document defining the general state of the art which is not considered to be of particular relevance</p> <p><sup>"E"</sup> earlier document but published on or after the international filing date</p> <p><sup>"L"</sup> document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p><sup>"O"</sup> document referring to an oral disclosure, use, exhibition or other means</p> <p><sup>"P"</sup> document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p><sup>"T"</sup> later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p><sup>"X"</sup> document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p><sup>"Y"</sup> document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p><sup>"&amp;"</sup> document member of the same patent family</p> </div> </div>										
<b>IV. CERTIFICATION</b> <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border-bottom: 1px solid black;">Date of the Actual Completion of the International Search</td> <td style="width: 50%; border-bottom: 1px solid black;">Date of Mailing of this International Search Report</td> </tr> <tr> <td style="text-align: center; height: 30px;">5th April 1991</td> <td style="text-align: center;">10.05.91</td> </tr> <tr> <td style="border-bottom: 1px solid black;">International Searching Authority</td> <td style="border-bottom: 1px solid black;">Signature of Authorized Officer</td> </tr> <tr> <td style="text-align: center;">EUROPEAN PATENT OFFICE</td> <td style="text-align: center;">             Alfredo Prein         </td> </tr> </table>			Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	5th April 1991	10.05.91	International Searching Authority	Signature of Authorized Officer	EUROPEAN PATENT OFFICE	 Alfredo Prein
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ON INTERNATIONAL PATENT APPLICATION NO. PCT/US 90/07348

SA 43377

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